THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

FEB 7 1974

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☒ Second Renewal ☐ Date: February 1, 1974

1. Principal Investigator (give title and degrees):

Charles G. Cochrane, M. D. - Member

2. Institution & address:

Scripps Clinic and Research Foundation
476 Prospect Street
La Jolla, California 92037

3. Department(s) where research will be done or collaboration provided:

Experimental Pathology

4. Short title of study:

The Mediation of Inflammatory Injury of Tissue

5. Proposed renewal date: July 1, 1974

6. How results to date have changed earlier specific research aims:

The enzymatic constituents of alveolar macrophages have been found to activate Hageman factor and with it, the intrinsic clotting, kinin forming and fibrinolytic systems. Our attention will now be directed toward analysis of the enzymes involved and their mechanism of release from the alveolar macrophages. In addition, Dr. John Craighead (personal communication) has observed kaolin in alveolar macrophages of smokers. Since kaolin is an excellent activator of Hageman factor, we will determine the relationship of aerosolyzed, inhaled kaolin, alveolar macrophages and pulmonary inflammation.

7. How results to date have changed earlier working hypothesis:

Earlier hypotheses have not changed.

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8. Any additional facilities now required? Describe briefly:

No new facilities are required.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

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10. Append outline of experimental protocol for ensuing year.
11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

1. Cochrane, C.G., Revak, S. D. and Wuepper, K. D., Activation of Hageman factor in solid and fluid phases. A critical role of kallikrein. J. Exp. Med., 138: 1564, 1973.
2. Wuepper, K. D., Prekallikrein deficiency in man. J. Exp. Med., 138: 1331, 1973.
3. Henson, P. M., Mechanism of release of granule enzymes from human neutrophils phagocytosing aggregated immunoglobulin: An EM study. Arthritis and Rheum., 16: 208, 1973.
4. Cochrane, C. G., Revak, S. D., Aikin, B. S. and Wuepper, K. D., The structural characteristics and activation of Hageman factor, In Inflammation, Mechanisms and Control, (Lepow and Ward, eds), Academic Press, 1972, p. 119.
5. Wuepper, K. D., Biochemistry and biology of components of the plasma kinin forming system, In Inflammation, Mechanisms & Control, (Lepow and Ward, eds), Acad. Press, 1972, p. 93.

(continued)

12. Summary progress report (append in standard form as separate document, unless recently submitted).

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11. Publications (continued)

6. Johnston, A., Cochrane, C. G. and Revak, S. D., The relationship of Pf/dil and activated human Hageman factor., J. Immunol., 112: 1974.

7. Ulevitch, R., Letchford, D. and Cochrane, C. G., A direct enzymatic assay of activated Hageman factor, Thrombosis et Diath. Hemorr., March, 1974.

8. Russell, S. and Cochrane, C. G., Cellular events associated with regression and progression of murine (Moloney) sarcoma in mice. Int'l J. Cancer, March, 1974.

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10. Appended outline of experimental protocol for ensuing year.

A. Experimental activation of the intrinsic clotting, kinin forming and fibrinolytic systems in lung. Alveolar macrophages will be isolated from lungs of rabbits by lavage (Brain et al. Arch. of Int. Med. (26:477, 1970), a technique already employed in this laboratory, and the granules obtained by disruption of the cells in hypertonic sucrose. After lysis of the granules, the constituents will be assayed for their capacity to activate Hageman factor over a range of conditions of pH, temperature and ionic strength. Since Hageman factor,activated by enzymes such as plasmin or kallikrein is cleaved in the process, a similar effect of the granular constituents of alveolar macrophages will be examined. This will define the mechanism of activation as fluid phase rather than solid phase (i.e., when Hageman factor binds to negatively charged surfaces such as that provided by alveolar membranes). The implication would be that clotting of plasma, and release of kinins and plasmin would occur in the fluid of the alveolar spaces, thereby provoking a diffuse reaction.

The granular constituents of the alveolar macrophages will then be fractionated on DEAE Sephadex A50 under conditions of constant pH and varying salt concentration. Enzymes or other activators of Hageman factor will be measured and isolated and identified through additional chromatographic and physical methods.

To determine the rate and extent of activation of Hageman factor and its related components (prekallikrein, clotting factor XI and plasminogen), these proteins will be radiolabelled and injected intravenously in rabbits. The disappearance from the circulation and accumulation in alveolar tissues and lavage-washings will be measured quantitatively. By SDS acrylamide gel electrophoresis under reducing conditions, activation of the Hageman factor, prekallikrein, plasminogen and factor XI will be determined since the molecules are cleaved during fluid phase activation (J. Exp. Med., 138: 1564, 1973). These measurements will be made in rabbits receiving various forms of pulmonary injury: 1) rabbits will be injected with goat antibody directed specifically to basement membrane of lung to induce injury; 2) rabbits will be exposed to aerosolyzed finely particulate kaolin. Dr. Paul Gross has expressed willingness to help in these experiments; 3) immunologic complexes of bovine-albumin(BSA)-anti BSA will be prepared in an excess of antigen (J. Exp. Med., 118: 489, 1963) and injected intravenously. These complexes deposit in the pulmonary vasculature and induce acute inflammation involving complement, neutrophils and platelets; 4) IgE mediated active systemic anaphylaxis will be produced in rabbits which causes release of vasoactive amines both intravenously and from the mast cells of the pulmonary beds.

In each of these experimental conditions the following examinations will be performed:

1. Careful histologic examination of the inflammatory reaction in the lung.
2. Fluorescent antibody localization of Hageman factor and kallikrein in the tissues. The presence or absence of fibrin will also be determined.
3. Kinetic analysis of the disappearance of radiolabelled components from the circulation as noted above.

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4. Accumulation of radiolabelled components in the pulmonary bed as compared with accumulation in other organs.

5. Analysis of the component molecules with SDS acrylamide gel electrophoresis to detect cleavage and thereby evidence of fluid phase activation.

These experiments are directed at the mechanism of activation of Hageman factor and its related systems in pulmonary inflammatory disease. Solid phase (localized) activation of Hageman factor will be detected by fluorescent antibody techniques and fluid phase (diffuse) activation by analysis of cleaved Hageman factor in the lavage fluid. It must be emphasized that these events may be of great importance in the injury of lung, the deposition of fibrin and stimulation of collagen formation. Perhaps the best studied parallel to this series of events is found in glomerulonephritis where sclerosis of the glomerulus may be directly related to the deposits of fibrin in Bowman's space.

B. Activation of Hageman factor and its related systems by human alveolar macrophages.

1. Using alveolar macrophages obtained from the pulmonary division of the Department of Medicine, University of California, San Diego, constituents capable of activating members of the Hageman factor systems will be sought along lines similar to those noted above in the rabbit studies. The cells will be provided by Drs. J. Clausen and Anthony Catanzarro under collaborative studies already established.

2. Following the observation by Craighead and his associates, we will determine if kaolin particles are present in the macrophages by electron microscopy and if the presence of kaolin is associated with smoking. According to Craighead, a potential source of the kaolin is the tobacco leaves which are apparently dried with kaolin as a drying agent. The macrophages will be lysed in NaOH, and the particles isolated and tested for their ability to bind and activate ^{125}I human Hageman factor. This is a property of kaolin that can be measured with exquisite sensitivity. Hageman factor already bound to the particles obtained from the macrophages will be sought in addition, although the liklihood of this is poor since the phagocytized particles would almost certainly lose bound proteins.

In addition, normal alveolar macrophages will be exposed to kaolin in the presence of ^{125}I Hageman factor. Release of activated, cleaved Hageman factor will be measured using acrylamide gel electrophoresis in order to determine the extent of fluid phase activation of Hageman factor occurring during the phagocytic process. This will shed light on potential diffuse release of injurious mediators.

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12. Summary progress report.

1. Structural studies of Hageman factor, the first component of the intrinsic clotting, kinin forming and fibrinolytic systems.

Hageman factor was found to be activated by two mechanisms. The first, occurring in solid phase is induced by molecular conformational changes associated with the binding of the molecule to negatively charged surfaces. Such insoluble materials as kaolin or vascular basement membranes and collagen serve to bind and activate Hageman factor. Preliminary evidence indicates that cut sections of pulmonary tissue also bind Hageman factor. In the second method of activating Hageman factor, enzymes derived from plasma and tissue serve to activate the molecule in fluid phase. Kallikrein, plasmin, factor XI and enzymes derived from alveolar macrophages were all found active in this regard. In fluid phase activation, Hageman factor is cleaved into fragments of 52,000, 40,000 and 28,000 MW. The 28,000 MW fragment bears enzymatic activity, and in turn is responsible for the activation of prekallikrein and factor XI of the intrinsic clotting system.

In order to obtain significant activation of the three systems, prekallikrein was found to be essential. In its absence, Hageman factor in plasma would not trigger the intrinsic clotting and fibrinolytic system to a degree adequate for measurement. These studies were made possible by the identification of a plasma obtained from individuals genetically deficient in prekallikrein - the so-called Fletcher trait plasma. Studies in this laboratory identified the missing factor as prekallikrein (1, 2).

2. Mechanisms of release of granule enzymes from human neutrophils phagocytosing aggregated immunoglobulin.

The reaction of human neutrophils with immunologic complexes or aggregates was found to depend upon specific surface receptors. If the aggregates were of a suitable size, phagocytosis ensued: particles were engulfed and granules released into the phagocytic vacuole. A proportion of potentially injurious granule constituents, however left the neutrophil through mechanisms which do not involve cell lysis but appear to be a consequence of the normal degradation phenomenon. Thus, if for any reason the vacuole was, or later became, open to the outside, there was extracellular release of the granules' enzymes. By means of electronmicroscopy, four possible mechanisms were demonstrated which might morphologically account for the escape of vacuolar contents from human neutrophils phagocytosing aggregated γ-globulin. According to the data, there was no release of lactic dehydrogenase and the cells were not lysed. Extrusion of granules seemed to occur primarily into vacuoles. Such vacuoles, however, occasionally had direct access to the exterior, as for example: (1) when they apparently opened to allow additional material to be ingested; (2) when they remained connected to the exterior by a narrow slit; (3) when two cells were involved in phagocytosis of a single particle (aggregate); (4) when extrusion of granules preceded the complete closing of pseudopods around the aggregates.

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13. Budget for the coming year:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Charles G. Cochrane	20	-0-
Peter M. Henson	20	-0-
Stephen W. Russell	50	9,000
Technical		
Part time services of animal caretaker and dishwasher		2,500
	Sub-Total for A	$11,500
B. Consumable supplies (by major categories)		
Chemicals, proteins		1,000
Glassware, plasticware		900
Animals, feed and bedding		1,000
	Sub-Total for B	2,900
C. Other expenses (itemize)		
none		---
	Sub-Total for C	---
	Running Total of A + B + C	$14,400
D. Permanent equipment (itemize)		
none		---
	Sub-Total for D	---
E. Indirect costs (15% of A+B+C)	E	$2,160
	Total request	$16,560

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14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Immunologic Studies	United States Public Health Service	$150,000	1974-1975
The Mediation of Inflammatory Injury of Tissues	Council for Tobacco Research	$16,500	1973-1974

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

O. K. Kincaid, Jr.

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Principal investigator

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Signature Charles G. Cochrane Date Feb. 2, 1974

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Responsible officer of institution

Typed Name Frank J. Dixon, M. D.

Title Chairman, Biomedical Research Departments

Signature Frank J. Dixon Date Feb. 4, 1974

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